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Soil Bioremediation in Heavy Metal Contaminated Mining Areas: A Microbiological/Biotechnological Point of View

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Authors' contributions

This work was carried out in collaboration between both authors. Author JDV performed the soil sampling and the laboratory experiments and wrote the first draft of the manuscript. Author VMS designed the study, managed the literature searches, supported the laboratory activities and wrote the last draft of the manuscript. Both authors read and approved the final version of the manuscript.

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ABSTRACT

Bioremediation concerns the use of plants and microorganisms or their parts, for the decontamination and recuperation of polluted areas. The improvement of the bioremediation techniques is possible due to the last decade advances of the microbiological and biotechnological knowledge and methods. This study aimed to shortly review and discuss the bioremediation of mining areas contaminated with heavy metals focusing in some microbiological and biotechnological techniques. Strategies considered to be important on further studies are also presented.

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1. INTRODUCTION

The study of heavy metal deposition and accumulation in soil is of increasing interest because of the awareness that heavy metals present in soils may have negative consequences on human health and on the environment. The heavy metals most frequently found at contaminated sites are lead, chromium, arsenic, zinc, cadmium, mercury, nickel and copper [1]. Soils may become contaminated by the accumulation of heavy metals and metalloids from different sources such as emissions from industrial areas, disposal of high metal wastes, leaded gasoline and paints, application of fertilizers, animal manures, sewage sludge, pesticides, coal combustion residues, spillage of petrochemicals, and atmospheric deposition. Immobilization, soil washing, and bioremediation techniques are frequently listed among the best-demonstrated available technologies for remediation of heavy metal-contaminated sites [1]. Bioremediation refers to the use of biological processes for the clean-up of contaminated land and water, usually groundwater. It consists on a group of applications, which involve the detoxification of hazardous substances by means of microbes and plants, instead of transferring them from one substrate to another.

There are several technologies in common use for bioremediation, divided broadly between *ex situ* and *in situ* methods. *Ex situ* technologies usually involve the construction of windrows or biopiles, either on site or at a remote location. *In situ* technologies are much less obtrusive, involve significantly fewer earthworks, but also require longer treatment times and suffer from a lack of control compared to *ex situ* technologies [2]. Currently, biotechnological tools in association with microbiological techniques have enlarged the battery of possibilities in bioremediation. For instance, the stimulation of specific gene expression and the use of particular microbial strains [3] are clear clues about the potential of using biotechnological and microbiological tools in the bioremediation of soils contaminated with heavy metals.

Traditionally, the field of microbiology has been concerned with and focused on studies at the microbial community and/or population levels. Information on how cells respond to their environment, interact with each other, or undergo complex processes such as cellular differentiation or gene expression has been obtained mostly by inference from community or population-level data. More recently, new appreciation for the existence and importance of cellular heterogeneity, coupled with the latest advances in technology, has driven the development of new tools and techniques for the study of individual microbial cells [4]. Among these technological advances, the genetic engineering can be used to improve the degradation of hazardous wastes under laboratory conditions by creating genetically modified organisms [5].

Recombinant bacteria can be obtained by recombinant DNA techniques or by natural genetic exchange between bacteria. Using the suitable biotechnological tools, appropriate genes can be inserted into a specific microorganism, aiming the production of particular enzyme, which can, for example, degrade various pollutants [6]. In this perspective, the use of genetically engineered microorganisms represents a research frontier with broad implications. These genetically engineered microorganisms can be tested under laboratory conditions and then be used in bioremediation enterprises, since they have potential benefits for removal of different contaminants from soil, groundwater and activated sludge environments, exhibiting enhanced degradative capabilities encompassing a wide range of chemical contaminants [7]. Although microorganisms are not able to degrade heavy metals, genetically modified bacteria or fungi may increase the migration and transformation of such pollutants through the enhancement of its capability of changing physical and chemical characteristics of the contaminants.

In this study, we aimed to assess and discuss the main microbiological and biotechnological tools employed in the bioremediation of mining areas contaminated by heavy metals, with examples from copper mining areas. We also present some results of our ongoing study on bioremediation of a copper mining area in Southern Brazil with view on four basic strategies that are considered most important to be the focus on further studies in this subject.

2. SOIL CONTAMINATION IN MINING AREAS: THE COPPER MINES AS AN EXAMPLE

Mining activities have a considerable impact on the environment. In addition to the local disturbance of the soil profile and structure, a more widespread contamination of soils, vegetation and watercourses by high concentrations of metals and metalloids can occur [8]. When present in water or soil, heavy metals may be introduced into food chains and thus cause irreversible damage to animals and humans [9,10,11]. Within the domain zones of mining sites, both, the soil and the mine-drainage waters may contain significant amounts of heavy metals [8,12]. Moreover, daily activities in mine areas such as excavation, ore transference, smelting, and refining, discharge large amounts of spoils, effluents, wastewater and dust containing heavy metals into the soil in the neighborhood.

Copper (Cu) is an essential element for all living beings, since it acts as co-factor in several enzymatic reactions. Despite of that, exposition to excessive levels of this metal may lead to different metabolic problems. Short-term exposure to drinking water contaminated with copper ions may cause gastrointestinal distress, while long-term consumption may cause liver or kidney damage [9]. Although agricultural activity is the main source of soil contamination with copper [13], very high levels of contamination with this heavy metal are found in copper mine tailing soils [14], where the Cu concentration in topsoil (0–30 cm) can be as high as several thousand $mg.kg^{-1}$. While a range from 13 to 24 $mg.kg^{-1}$ of Cu is naturally found in soil [15], the concentration of copper found in soil from mining areas in India, China, Iran, Spain, South Korea, Vietnam, Morocco and Peru ranged from 63.49 to 5270.0 $mg.kg^{-1}$ [8,16,17]. The main points of soil contamination are the areas of mineral extraction (Fig. 1A-B and 1E-F) and of reject deposition (tailings dam, where the discards of the mineral extraction are stored; Fig. 1C-D).

In addition to copper, other metals contaminate the area around copper mines. The types of metal contaminating such areas depend on the composition of the mined copper ore and the accompanying gangue [8]. The Camaquã Mines in Southern Brazil (30°47'S, 52°24'W) comprehend a complex of open pit and underground mining which was exploited from the years 1939 to 1996 and produced about 10,000 tons of copper per year in the last period of operation. After finishing the operation, the area was abandoned and no recuperation or remediation initiatives were performed up to now. The characterization of the contaminant heavy metals in the superficial waters around the operation area revealed that mining activities produced partition changes in most of the heavy metals studied (Cu, Pb, Zn, Cd, As, Mn, Al and Fe), reducing the residence time and availability of Cu and Zn, while Mn, Cd, As and Pb had these parameters increased [18]. Concerning the soil of the tailings dam, the levels of copper ranged from 0.034 to 0.08%, while the level of soluble iron was 0.003 $mg.L^{-1}$, revealing quite low deposition of these metals [19].

Despite the low level of contamination observed in the Camaquã Mines, the cumulative nature of heavy metals is still a health concern and remediation of this area is needed. In general, we can found microorganisms able to survive in the most extreme environmental conditions, including the soil of mining areas with high concentration of heavy metals. Different bacterial species have been characterized in areas contaminated with toxic compounds, as *Pseudomonas* sp and *Bacillus* sp [20,21,22,23, 24,25], and may be used in programs of bioremediation. In addition to these bacteria, other species may be choose, depending on the main characteristics of the area to be recovered through bioremediation. For instance, microorganisms able to perform symbiosis with the root system of tree species may be of interest for a rhyzoremediation/dendroremediation system aiming soil and landscape recovery of mining areas.

3. BIOREMEDIATION OF AREAS CONTAMINATED WITH HEAVY METALS

The bioaccumulation of heavy metals causing toxicity in human, animals, microorganisms and plants is an important issue for environmental health and safety. In recent years, several approaches for bioremediation have been proposed as alternatives for weakening this problem.

Bech et al. [8] investigated the concentration of copper, zinc, manganese, aluminum and iron in plants growing in different sites in a copper mine region in Peru and suggested further investigations to confirm the tolerance of the sampled plant species for high heavy metal

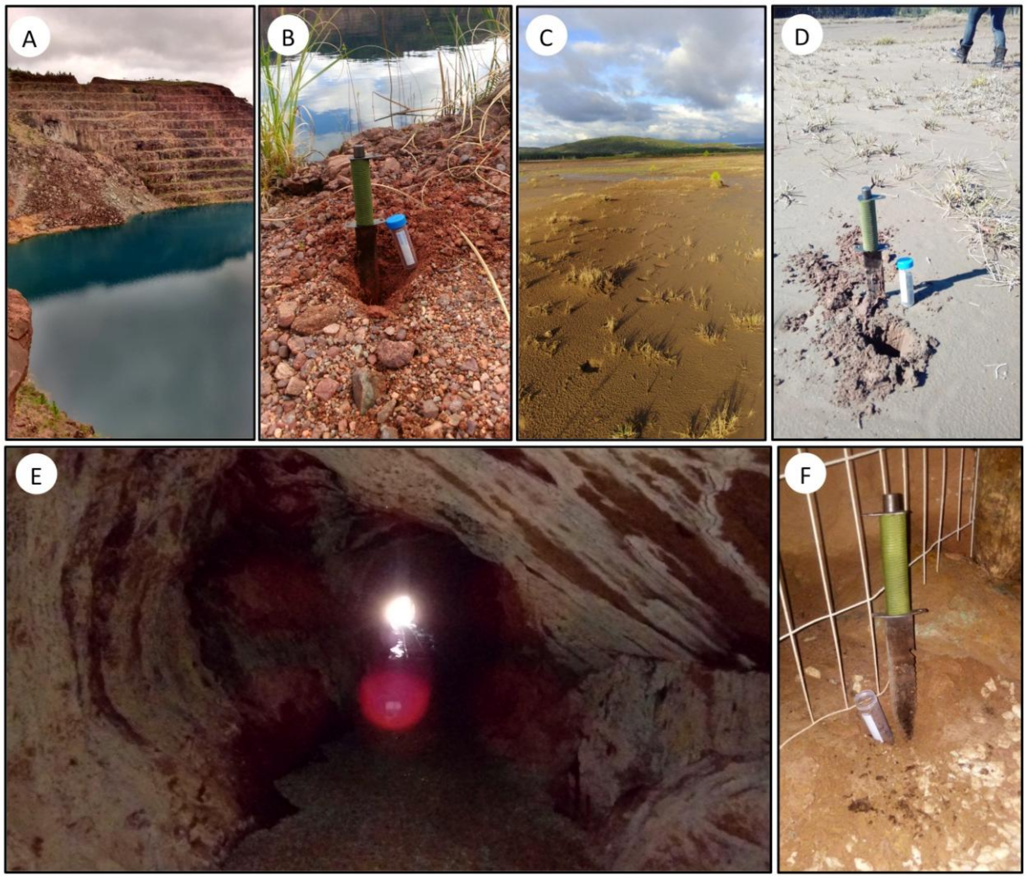

Fig. 1. Mining areas of copper in Camaquã Mines, Southern Brazil (30°47'S, 52°24'W). (A) View of the open pit mining area, where an artificial lake was formed by rainwater deposition. (B) Sample collection for physicochemical and microbiological analyses of the soil of open pit mining area. (C) Tailings dam. (D) Sample collection for physicochemical and microbiological analyses of the tailings dam's soil. (E) Inside view of the underground mine. (F) Sample collection for physicochemical and microbiological analyses of the underground mine's soil

contamination. The authors proposed that these species might be a very attractive material for the revegetation of several mine sites located at high altitudes in equatorial regions.

The potential role of arbuscular mycorrhizal fungi associated with native plants from China in promoting revegetation of copper mine tailings was evaluated by Chen et al. [21]. The experiment evidenced the beneficial impacts of mycorrhizal colonization on plant growth and provided evidences for the potential use of local plant species in combination with arbuscular mycorrhizal fungi for ecological restoration of metalliferous mine tailings.

In Southeastern Brazil, Perlatti et al. [26] evaluated the possible implications for the use of plant species in restoring a copper mining area by calculating the balance between the copper mobilized in the rhizosphere and the copper absorbed by the plants. The results showed that none of the four evaluated plant species fulfilled the requirements for a classification as hyperaccumulator species (i.e. >1000 $mg.kg^{-1}$ Cu in shoots or ratio between Cu in leaves and soil >1). However, two species showed the characteristics of immobilizing species (the ratio between the mobilized Cu in the rhizosphere and the Cu accumulated in their tissues was negative, indicating that these species can uptake more Cu than that is mobilized by their rhizospheric processes, decreasing the amount of bioavailable or potentially bioavailable copper in the environment), being suitable for remediation and/or revegetation of copper contaminated areas.

The use of genetically modified strains of *Saccharomyces cerevisiae* for the absorption of environmental copper was proposed by Geva et al. [3]. The human genes MT2 and GFP-hMT2 were introduced into the yeast cells aiming to enhance the ability of this microorganism for copper ion bioremediation.

As a more general trend, bioremediation methods include at least 10 different strategies, as shown in Table 1 [27].

Table 1 shows that among the different strategies commonly employed in bioremediation of contaminated soil, at least 60% concerns the application of microbes (bacteria or fungi) as the main tool. Microbes can be used alone or associated with other organisms, mainly plants. More recently, the possibility of using the stimulation of gene expression and the genetic transformation of microorganisms prompted the search for further alternatives [3].

4. MICROBIOLOGICAL AND BIO-TECHNOLOGICAL TOOLS FOR BIOREMEDIATION

The use of microbes for the production of different compounds of human interest is not a new task. It goes back to the Egyptian and Roman ancient civilizations, which employed yeast for manufacturing beer, wine and bread. With the advance of the modern biotechnology, which employs the gene manipulation as one of the main tools for reaching new services and products, the number of possible uses for microbes enlarged extensively, including bioremediation of heavy metal-contaminated areas.

Bioremediation of heavy metal-contaminated areas by growing microbial cells may lead to simultaneous removal of toxic metals and other inorganic and organic contaminants. Metal removal by cells possessing high tolerance to

Table 1. Bioremediation methods and their background [after 27]

Method	Background
Biosorption	The passive uptake of pollutants by biological materials as dead microbial and renewable agricultural biomass.
Biostimulation	Injection of nutrients and other supplementary components to the native microbial population, stimulating living microbial population to propagate at a hastened rate.
Mycoremediation	Stimulating living fungi/mycelial to degrade or sequester contaminants in the environment, to repair or restore the weakened immune system of environment, or to promote ultrafiltration.
Rhizoremediation	Enhancement of the metal extraction process through the root system of plants by its association with microbes.
Genoremediation	Enhancement of the capacity of metal accumulation and tolerance by over-expressing natural or modified genes encoding enzymes of interest for bioremediation.
Biomineralization	Natural synthesis of structured inorganic materials (minerals) by living organisms from metal contaminants of the soil.
Phytostabilization	Minimization of the mobility of heavy metals in polluted soils using the plant roots and microbial interaction through the immobilization of contamination by binding the pollutants to soil particles in the rhizosphere of woody species.
Hyperaccumulation	Exceptional accumulation of pollutants in plants naturally tolerant to heavy metals.
Dendroremediation	Growing trees with high metal resistance potential, in polluted soils in order to remove, sequester or chemically decompose the pollutants.
Cyanoremediation	Stimulating cyanobacteria (either wild type, mutant or genetically engineered forms) for remediation of polluted areas through accumulation, degradation or absorption the pollutants.

various metals can combine biosorption (binding and concentration of metal ions by dead cells), bioprecipitation (process of formation of mineral phases, the bioprecipitates or biominerals, by organisms), and the metabolic uptake of metals [3; see Table 1]. Another mechanism is the bioaccumulation, which depends on metabolism of the microorganism and is related to transportation of metal ions through the cell membrane with their subsequent intracellular accumulation [28,29].

Aiming to expand the efficiency of microorganisms as bioremediation tools, the gene manipulation can be used in order to induce the overproduction of proteins responsible for binding metal ions in the cytoplasm [28,30, 31,32,33], as well as to introduce selected genes in particular microbial strains [3].

Genetic engineering has the potential to improve or redesign microorganisms, where biological metal-sequestering systems will have a higher intrinsic capability as well as specificity and greater resistance to environmental conditions. Higher organisms respond to the presence of metals, with the production of cysteine-rich peptides, such as glutathione, phytochelatins and metallothioneins, which can bind and sequester metal ions in biologically inactive forms. The overexpression of metallothioneins in bacterial cells will result in enhanced metal accumulation, thus offering a promising strategy for the development of microbial-based biosorbents for the remediation of metal contamination [33]. However, the perception of environmental biotechnology by the general public may trouble field applications of genetically modified microorganisms [34], requiring well-grounded studies on this issue.

Moreover, there is a need of evaluation of the response of bacteria, fungi or algae to the environmental conditions of the soil for their use as tools for bioremediation also for non-genetically modified microorganisms. For instance, the *in vitro* culture of *Mesorhizobium loti* under different pH conditions and carbon sources revealed differential production of exopolysacharides (important compounds in the process of bioremediation with bacteria) by two different strains of this symbiotic microorganism [35]. Similarly, differential growth was observed for *Mesorhizobium loti* and *Bradyrhizobium japonicum* cultured *in vitro* using different pH conditions and sources of carbon [36].

5. TAKING DECISIONS ON SOIL BIOREMEDIATION

For soils contaminated with heavy metals, the physical and chemical form of the contaminant strongly influences the selection of the appropriate remediation treatment approach. In addition, information about the microorganisms surviving in such environmental and the physical and chemical characteristics of the soil are also very important information for taking such decision. Thus, information about the microorganisms, the physical characteristics of the area and the type and level of contamination at the site must be obtained to enable accurate assessment of contamination and corrective alternatives of the region to be recovered [1].

The choice of the most appropriated microorganism to be employed in bioremediation enterprises has to take in consideration the study region. The simplest strategy for identifying and isolating bacterial species with potential for bioremediation in such areas is the culture of sample soils in selective medium with heavy metal. This strategy was employed by our group in the abandoned area of the Camaquã mines, searching for bacteria with the capacity of surviving in such extreme conditions. Sample soils were grown *in vitro* under different concentrations of copper in the medium, aiming to select the species able to survive in these conditions (Fig. 2A). Such bacteria can be characterized concerning its bioaccumulating proprieties. Bacteria surviving under extreme conditions of heavy metal contamination may then be directly tested as bioremediators or be source of genes to be transferred to other bacterial species more adapted for the desired propose (e.g. concomitantly performing bioremediation and landscape recover through rhyzoremediation).

Since the metabolism of the microorganism also greatly interferes in the success of the bioaccumulation process [3], such characterization is also important. The capacity of growing under different environmental conditions [36] and the production of exopolysaccharides [35] are simple tests (Fig. 2B) that can be performed *in vitro* with basic laboratorial conditions (sterilized airflow chamber and temperature controlled environment at 28°C) and chemical reagents (yeast extract, dipotassium phosphate, magnesium phosphate, sodium chloride, calcium chloride, agar and carbon source, e.g. D-mannitol, sucrose or dextrose) to characterize the potential of particular bacterial strains for the bioremediation process.

In addition, parameters such as the pH (Fig. 2C), total humidity and particles density of the soil from the open pit mine region (Fig. 1A-B) and from the tailings dam (Fig. 1C-D) were characterized. Despite the apparent difference of both soils, the pH of two samples of the open-air mine was 9.26 and 9.02, while the pH measures for two samples of the tailings dam was 8.83 and 9.07. This information is quite important since in the soil, Cu strongly complexes to the organic compounds implying that only a small fraction of copper will be found in solution as ionic copper [6]. The solubility of Cu is drastically increased at pH 5.5 [37], which is rather close to the ideal farmland pH of 6.0–6.5. Thus, we know that the solubility of the copper in these areas is low, demanding specific strategies for such environment. Concerning the total humidity of the soil, the open pit mining samples revealed a much lower percentage (5.61–8.20%) in comparison to the tailings dam area (17.15–17.50%), although the particles density was also very similar among samples. The open pit mine region revealed a particles density ranging from 2.009 to 2.077 $g.cm^{-3}$, while for the tailings dam samples, the particles density ranged from 1.934 to 1.958 $g.cm^{-3}$. These simple results reveal the need of using strategies able to remediate a soil in which the copper will present low solubility and of considering the strong difference in humidity levels of each site.

While the physical and chemical characteristics observed in the Camaquã Mines is somewhat similar across sites concerning particles density and pH, soils from different sampling sites at a copper mine in Peru highly differed in texture and pH (pH values ranging from 3.33 to 5.86; [8]), suggesting the need of more distinct strategies to perform the bioremediation of this area.

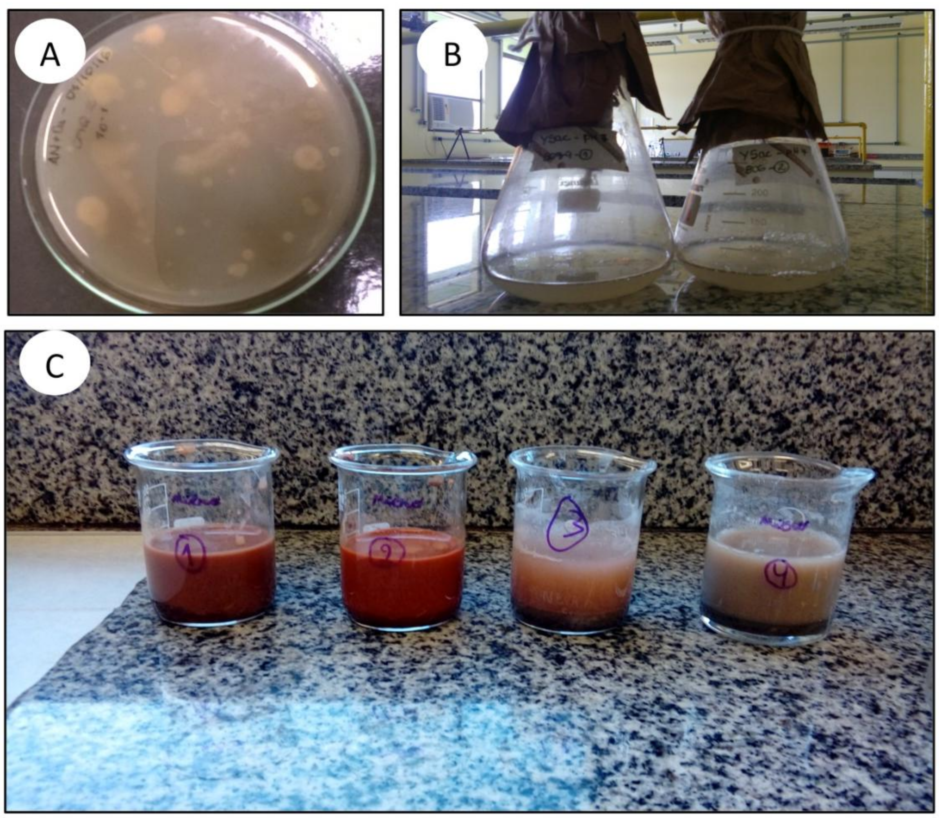

Fig. 2. Physicochemical and microbiological analyses of sampled soil in the mining region of Camaquã Mines, Southern Brazil. (A) *In vitro* growth in copper-enriched culture medium of bacterial species from soil sampled in the open-air mine region. (B) *In vitro* culture of bacterial strains of *Mesorhizobium loti* SEMIA806 and *Bradyrhizobium japonicum* SEMIA5099 in liquid medium. (C) Dilution of soil samples from the open-air mine region for pH analysis

6. PERSPECTIVES AND POTENTIAL ADVANCES

In comparison with most physicochemical solutions, bioremediation is a cost-effective technology that leads to absorption/accumulation or complete mineralization of contaminants through living beings, mainly microorganisms and plants [13]. The use of microorganisms (or microbiological tools) for the recovery of contaminated areas is an extremely important strategy and has been widely recognized as very promissory bioremediation method. As perspectives and potential advances for the use of microbiological and biotechnological tools for bioremediation we list four strategies that we consider the most important to be focus on further studies.

1. Taking into consideration the natural intra-specific variation on the upper limits of bacteria resistance to heavy metal concentration in the soil, the screening of such “hyper-resistant” microorganisms should be the start point for studies of mining soil bioremediation.
2. Considering that soil contamination in mining areas does not mean the occurrence of one single chemical compound, but usually a combination of different heavy metals, the investigation of the synergistic effect of bacterial mixtures on the bioremediation of such soils have to be deeper investigated.
3. Pondering the negative impact of the deforestation caused in degraded areas, the use of the combinations of bacteria and tree species have to be further studied, in order to enable the recovery of the soil and the recuperation of the landscape.
4. Bearing in mind the advances of the biotechnology in the last decades, genetically modified bacteria should be tested for bioremediation enterprises in different soils and for absorption, precipitation or accumulation of different heavy metals contaminating the environment.

Certainly, the forthcoming advances on microbiology and on biotechnology will quickly bring new promising strategies. Similarly, testing these four strategies will support or refute their viability for the proposed situations. However, the most important thinking is constantly search for future perspectives using the currently available microbiological and biotechnological alternatives.

7. CONCLUSION

The use of microorganisms as bioremediation tool has shown increased importance in the last decades. In addition, the microbiological and biotechnological advances occurred in the last years enables the development of easy and efficient strategies for mining soil bioremediation. Therefore, in addition to selecting hyper-resistant bacteria, the use of microbial genetic transformation is a quite powerful tool, which should be further exploited.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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